PATENT
Attorney Docket No. **046124-5005-02-US**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	
	)	
Takashi OSUMI *et al.*	)	**PRIOR APPLICATION:**
	)	
Application No.: To be Assigned	)	Group Art Unit: 1652
	)	
Filed: May 10, 2001	)	Examiner: E. Slobodyansky, Ph.D.
	)	
For: GREEN FLUORESCENT PROTEINS AND BLUE FLUORESCENT PROTEINS	)	

Commissioner for Patents
Washington, D.C. 20231
ATTN: BOX PATENT APPLICATION

Sir:

PRELIMINARY AMENDMENT

Prior to the examination on the merits, please amend this application as follows.

IN THE SPECIFICATION:

Please replace the paragraph at Page 6, lines 18-24 with the following rewritten paragraph:

In view of the above-mentioned problems, the present inventors performed extensive research and succeeded in the discovery of novel GFPs and BFPs that are free from such problems by introducing certain mutations into specific positions of the amino acid sequence for GFP or BFP, thus accomplishing this invention.

Please replace the heading at Page 12, line 16 with the following rewritten heading:

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Please replace the paragraph at Page 18, lines 13-21 with the following rewritten paragraph:

Moreover, methods for randomly introducing mutations are not particularly limited, and Mutagenic PCR as described below can preferably be used in this invention. The Mutagenic PCR can be carried out according to methods known in the art. (C.W. Dieffenback, ed. PCR PRIMER, A Laboratory Manual (Cold Spring Harbor Laboratory Press) (1995) pp. 583-588.) Concretely, the following conditions were employed in the examples.

Please replace the paragraph at Page 18, line 22 through Page 19, line 8 with the following rewritten paragraph:

About 50 ng of Plasmid BlueBFP (201) was added to 10 × mutagenic PCR buffer (70 mM $MgCl_2$, 500 mM KCl, and 100 mM Tris-HCl, pH 8.3 at 25 °C; 0.1% (w/v) gelatin) 10 µl, 10 × dNTP (2 mM dGTP, 2 mM dATP, 10 mM dCTP, and 10 mM dTTP) 10 µl, 10 mpol/µl primer (23mer M13Universal primer and M13Reverse primer) 3µ1, and H_2O 62µl, and mixed. Subsequently, 10 µl of 5mM $MnCl_2$ was added and mixed, and 1µl of Taq Polymerase (Takara) was added to conduct PCR (PC-700 available from ASTEC Inc. was used). The PCR was conducted in three tubes under the following conditions: 25 cycles at 94 °C for 1 min, 30 cycles at 45 °C for 1 min, and 35 cycles at 72 °C for 1 min, respectively.

Please replace Table 4 on Page 23, lines 5-20 with the following revised table:

TABLE 4

GFP						
101	none					
103			Phe64Leu			
104				Val163Ala	Ser175Gly	
105			Phe64Leu,	Val163Ala,	Ser175Gly	
BFP (as for BFP, the two mutations, Tyr66His (Y66H) and Tyr145Phe (Y145F), have been introduced into the sequence for GFP which serves as a basis)						
201	Y66H,	Y145F:				
202	Y66H,	Y145F:	Phe64Leu,			Leu236Arg
203	Y66H,	Y145F:	Phe64Leu			
204	Y66H,	Y145F:		Val163Ala,	Ser175Gly	
205	Y66H,	Y145F:	Phe64Leu,	Val163Ala,	Ser175Gly,	Leu236Arg

Please replace the paragraph at Page 25, line 19 through Page 26, line 19 with the following rewritten paragraph:

Unless otherwise so stated, CHO-K1 cells were grown in a F12+10% FBS medium in 5% CO_2 at 37 °C. The cells (1×10^5) were inoculated into a 6-cm dish, and on the following day, their transfection was conducted in two dishes as a pair by the calcium phosphate method. (C. Chen and H. Okayama Mol. Cell. Biol. 7: 2745-2752 (1987).) After transfection, the one dish was incubated at 37 °C and the other at 30 °C for 24 h. The transfected CHO cells were washed with 1 × PBS (-) three times, and they were dissolved in 1 ml of 10 mM Tris-HCl (pH 7.4)

containing 1% Triton X-100 and recovered in an Eppendorf tube. A supernatant (0.5 ml) from centrifugation at 3,000 rpm for 5 min was diluted 4-fold with the same buffer and fluorescence measurement was performed. Here, a pUcD2SRαMCS vector (empty vector) was transfected and used as a blank. A Hitachi F-2000 type fluorophotometer was used in the fluorescence measurement. In the measurement of GFPs, fluorescence was scanned between 460 nm and 600 nm at an excitation wavelength of 460 nm to measure the maximal value in the vicinity of the fluorescence wavelength of 510 nm. In the measurement of BFPs, fluorescence was scanned between 360 nm and 500 nm at an excitation wavelength of 360 nm to measure the maximal value in the vicinity of the fluorescence wavelength of 445 n.

Please replace the paragraph at Page 26, line 21 through Page 27, line 22 with the following rewritten paragraph:

The CHO cells were transfected with pUcD2SRαMCS (empty vector)(T. Tsukamoto et al. Nature. Genet. 11: 395-401 (1995)), phGFP(101)-Cl, phGFP(105)-Cl, phBFP(201)-CL, and phBFP(205)-Cl, respectively and grown at 37 °C and at 30 °C. Employing a sample prior to dilution as used in the fluorescence measurement previously described (8 μl), SDS-PAGE was performed on a 12% gel. With the use of a Horizonblot (ATTO Inc.), transfer was conducted onto a nitrocellulose membrane (Millipore Inc., HAHY394FO) under the conditions of 2 mA and 90 min per cm^2. After the membrane was taken out and washed with 1 × PBS, it was immersed in 1% skim milk/PBS and shaken at room temperature for 30 min. After the membrane was washed with 1 × PBS, it was immersed in 0.1% skim milk/PBS containing an anti-GFP antibody (Clonetech Inc.) that had been diluted 2,000-fold and shaken at 4 °C overnight. The membrane was washed with 1 × PBS for 5 min, and then with TPBS (0.05% Trion X-100/PBS) for 15 min three times. The membrane was immersed in 0.1% skim milk/PBS containing an anti-rabbit IgG antibody labeled with HRP (Amersham Inc.) that had been diluted 1,000-fold, and shaken at 4

°C for 1 h. The membrane was washed with 1 × PBS for 5 min, and then with TPBS (0.05% Trion X-100/PBS) for 15 min three times. The membrane was reacted with a chemiluminescence reagent (Amersham Inc. ECL) for 1 min, and then, was exposed to an X-ray film for 2 min.

IN THE CLAIMS:

Please substitute amended claims 8-14 for pending claims 8-14 and add new claims 15-20 as follows:

8. (Amended) DNA encoding a fluorescent protein comprising the amino acid sequence set forth in SEQ ID No. 1 with at least the mutations of Phe64Leu, Val163Ala, and Ser175Gly.

9. (Amended) DNA encoding a fluorescent protein comprising the amino acid sequence set forth in SEQ ID No. 1 with the three mutations of Phe64Leu, Val163Ala, and Ser175Gly.

10. (Amended) DNA encoding a fluorescent protein comprising the amino acid sequence set forth in SEQ ID No. 1 with at least the mutations of Tyr66His, Tyr145Phe, and Phe64Leu.

11. (Amended) DNA encoding a fluorescent protein comprising the amino acid sequence set forth in SEQ ID No. 1 with at least the mutations of Tyr66His, Tyr145Phe, Phe64Leu, and Leu236Arg.

12. (Amended) DNA encoding a fluorescent protein comprising the amino acid sequence set forth in SEQ ID No. 1 with the four mutations of Tyr66His, Tyr145Phe, Phe64Leu, and Leu236Arg.

13. (Amended) DNA encoding a fluorescent protein comprising the amino acid sequence set forth in SEQ ID No. 1 with at least the mutations of Tyr66His, Tyr145Phe, Phe64Leu, Val163Ala, Ser175Gly and Leu236Arg.

14. (Amended) DNA encoding a fluorescent protein comprising the amino acid sequence set forth in SEQ ID No. 1 with the six mutations of Tyr66His, Tyr145Phe, Phe64Leu, Val163Ala, Ser175Gly and Leu236Arg.

--15. DNA encoding a fluorescent protein comprising the amino acid sequence set forth in SEQ ID No. 1 with at least the mutations of Val163Ala and Ser175Gly.

16. DNA encoding a fluorescent protein comprising the amino acid sequence set forth in SEQ ID No. 1 with at least the mutations of Tyr66His, Tyr145Phe, Val163Ala and Ser175Gly.

17. A method of visually analyzing gene expression or protein localization in a cell comprising the steps of:

(a) providing a cell transfected with a vector comprising the DNA of any of claims 8, 9 or 15;

(b) culturing the cells under conditions to express a fluorescent protein encoded by the vector; and

(c) detecting the presence of the fluorescent protein.

18. The method of claim 17, wherein the cell is a mammalian cell.

19. A method of visually analyzing gene expression or protein localization in a cell comprising the steps of:

(a) providing a cell transfected with a vector comprising the DNA of any of claims 10, 11, 12, 13, 14 or 16;

(b) culturing the cells under conditions to express a fluorescent protein encoded by the vector; and

(c) detecting the presence of the fluorescent protein.

20. The method of claim 19, wherein the cell is a mammalian cell.--

REMARKS

After entry of the Preliminary Amendment, claims 8-20 are pending in this application.

I. Amendments to the Specification

The amendment on page 1 complies with Applicants' obligations under 35 U.S.C. § 120 to claim priority in the first paragraph of the specification.

The amendments to the specification on pages 6, 12, 18, 26 and 27 are typographical in nature, are made to place the application in better form for allowance, and do not add prohibited new matter.

The amendment to page 23 clarifies the nomenclature used in the table. Support for this change is presented in the specification on page 10, lines 20 and 25 and on page 11, line 4, and would be commonly understood by the skilled artisan. No prohibited new matter is added.

II. Amendments to the Claims

The amendments to the claims merely redraft the claims in independent form (claims 8-16) or correct the referenced dependency to the renumbered claims.

In addition, the word "gene" has been replaced by the word "DNA (claims 8-14), support for which is found at page 17, lines 12-16 and page 17, lines 26 to page 18, line 10.

The acronyms "BFP" or "GFP" have been replaced by the term "fluorescent," as was done for the allowed claims (U.S. Patent 6,194,548) to eliminate redundant terminology and is supported by the disclosure at page 1, lines 6-7 and page 6, lines 1-2.

No prohibited new matter is added by these amendments or claims.

III. Claim for Priority

Under the provisions of Section 119 of 35 U.S.C., Applicants hereby claim the benefit of the filing date of Japanese Patent Application No. 10-026418, filed January 23, 1998.

In support of Applicants' claim for priority, Applicants rely on the certified copy of Japanese Patent Application No. 10-026416 filed in United States Patent Application Serial No. 09/121,539, parent application to the instant divisional application.

CONCLUSION

For the reasons set forth above, entry of the foregoing amendment and examination of the application respectfully is requested. If there are any other filing or claim fees due in connection with the filing of this preliminary amendment, please charge the fees to our Deposit Account No. 50-0310. If a fee is required for any extension of time under 37 C.F.R. § 1.136 not accounted for above, such an extension is requested and the fee should also be charged to our Deposit Account.

The Examiner is invited to telephone either of the undersigned if a discussion might expedite placing the claims in condition for allowance.

Respectfully submitted,

MORGAN, LEWIS & BOCKIUS LLP

By: _____

Thomas F. Poché
Reg. No. 45,017

Date: May 10, 2001

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Specification:

Paragraph at page 6, lines 18-24 has been amended as follows:

In view of the above-mentioned problems, the present inventors [~~made extensive researches~~] performed extensive research and succeeded in the discovery of novel GFPs and BFPs that are free from such problems by introducing certain mutations into specific positions of the amino acid sequence for GFP or BFP, thus accomplishing this invention.

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DESCRIPTION OF THE [~~PREFRED~~] **PREFERRED** EMBODIMENTS

Paragraph at page 18, lines 13-21 has been amended as follows:

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Paragraph at Page 18, line 22 through Page 19, line 8 has been amended as follows:

About 50 ng of Plasmid BlueBFP (201) was added to [~~10xmutagenic~~] 10 × mutagenic PCR buffer (70 mM $MgCl_2$, 500 mM KCl, and 100 mM Tris-HCl, pH 8.3 at 25 °C; 0.1% (w/v) gelatin) 10 µl, [~~10xdNTP~~] 10 × dNTP (2 mM dGTP, 2 mM dATP, 10 mM dCTP, and 10 mM dTTP) 10 µl, 10 mpol/µl primer (23mer M13Universal primer and M13Reverse primer) 3µl, and H_2O 62µl, and mixed. Subsequently, 10 µl of 5mM $MnCl_2$ was added and mixed, and 1µl of Taq Polymerase (Takara) was added to conduct PCR (PC-700 available from ASTEC Inc. was used). The PCR was conducted in three tubes under the following conditions: 25 cycles at 94 °C for 1 min, 30 cycles at 45 °C for 1 min, and 35 cycles at 72 °C for 1 min, respectively.

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105			Phe64Leu,	Val163Ala,	Ser175Gly	
BFP (as for BFP, the two mutations, [~~Y66H~~] Tyr66His (Y66H) and [~~Y145F~~] Tyr145Phe (Y145F), have been introduced into the sequence for GFP which serves as a basis)						
201	Y66H,	Y145F:				
202	Y66H,	Y145F:	Phe64Leu,			Leu236Arg
203	Y66H,	Y145F:	Phe64Leu			
204	Y66H,	Y145F:		Val163Ala,	Ser175Gly	
205	Y66H,	Y145F:	Phe64Leu,	Val163Ala,	Ser175Gly,	Leu236Arg

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measure the maximal value in the vicinity of the fluorescence wavelength of 510 nm. In the measurement of BFPs, fluorescence was scanned between 360 nm and 500 nm at an excitation wavelength of 360 nm to measure the maximal value in the vicinity of the fluorescence wavelength of 445 n.

Paragraph at Page 26, line 21 through Page 27, line 22 has been amended as follows:

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In the Claims:

Claim 8 has been amended as follows:

8. (Amended) ~~[A gene]~~ DNA encoding ~~[the GFP]~~ a fluorescent protein ~~[according to claim 1]~~ comprising the amino acid sequence set forth in SEQ ID No. 1 with at least the mutations of Phe64Leu, Val163Ala, and Ser175Gly.

Claim 9 has been amended as follows:

9. (Amended) ~~[A gene]~~ DNA encoding ~~[the GFP]~~ a fluorescent protein ~~[according to claim 2]~~ comprising the amino acid sequence set forth in SEQ ID No. 1 with the three mutations of Phe64Leu, Val163Ala, and Ser175Gly.

Claim 10 has been amended as follows:

10. (Amended) [~~A gene~~] DNA encoding [~~the BFP~~] a fluorescent protein [~~according to claim 3~~] comprising the amino acid sequence set forth in SEQ ID No. 1 with at least the mutations of Tyr66His, Tyr145Phe, and Phe64Leu.

Claim 11 has been amended as follows:

11. (Amended) [~~A gene~~] DNA encoding [~~the BFP~~] a fluorescent protein [~~according to claim 4~~] comprising the amino acid sequence set forth in SEQ ID No. 1 with at least the mutations of Tyr66His, Tyr145Phe, Phe64Leu, and Leu236Arg.

Claim 12 has been amended as follows:

12. (Amended) [~~A gene~~] DNA encoding [~~the BFP~~] a fluorescent protein [~~according to claim 5~~] comprising the amino acid sequence set forth in SEQ ID No. 1 with the four mutations of Tyr66His, Tyr145Phe, Phe64Leu, and Leu236Arg.

Claim 13 has been amended as follows:

13. (Amended) [~~A gene~~] DNA encoding [~~the BFP~~] a fluorescent protein [~~according to claim 6~~] comprising the amino acid sequence set forth in SEQ ID No. 1 with at least the mutations of Tyr66His, Tyr145Phe, Phe64Leu, Val163Ala, Ser175Gly and Leu236Arg.

Claim 14 has been amended as follows:

14. (Amended) [~~A gene~~] DNA encoding [~~the BFP~~] a fluorescent protein [~~according to claim 7~~] comprising the amino acid sequence set forth in SEQ ID No. 1 with the six mutations of Tyr66His, Tyr145Phe, Phe64Leu, Val163Ala, Ser175Gly and Leu236Arg.